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High-contrast epi-fluorescence wide-field imaging of biological cells using integrating-bucket method

K.S. Park [a], W.J. Choi [b], J.B. Eom [c], K.S. Chang [a], B.H. Lee [d,*]

[a] Devision of Scientific Instrumentation, Korea Basic Science Institute (KBSI), 169-148 Gwahak-ro, Yuseong-gu, Daejeon 305-806, South Korea
[b] University of Washington, Department of Bioengineering, 3720 15th Avenue NE, Seattle, WA 98195, USA
[c] Korea Photonics Technology Institute, 5 Cheomdan 4 gil, Buk-gu, Gwangju 500-779, South Korea
[d] School of Information and Communications, Gwangju Institute of Science and Technology (GIST), 261 Cheomdan-gwagiro, Buk-gu, Gwangju 500-712, South Korea

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ABSTRACT

We present an optical lock-in detection scheme, called the integrating-bucket technique, as a signal-to-background ratio enhancement method for wide-field fluorescence imaging of biological cells. The proposed method uses sinusoidally modulated illumination light and captures four frames of fluorescence images, one per each quarter of the modulation period, by integrating the fluorescence intensity signal. The capability of this technique is demonstrated by imaging fluorescent bead solutions as well as labeled cells. The results show that the method yields a 4–10 dB higher signal contrast than conventional fluorescence microscopy, and a background-free fluorescence image can be extracted within a sub-second time scale. Our findings indicate that the proposed method could be advantageous for the long-term study of live cells.

1. Introduction

The use of fluorescence for biological imaging is a simple but very powerful method for visualizing protein distribution within cellular organelles. Fluorescence-contrast imaging has greatly aided biologists in understanding the complex biological processes involved in protein interaction dynamics, which is important for cell proliferation [1]. For cellular imaging, synthetic fluorescent dyes have been mainly used to enhance fluorescence contrast and identify the region of interest within a sample. However, there are several aspects of synthetic dye implementation that require improvement, such as low uptake efficiency, which restricts the contrast in fluorescence imaging. Photobleaching of fluorophores also hinders the long-term study of labeled cells. The staining rate can be increased by increasing the dye concentration, but this may adversely affect the cell viability owing to possible toxicity of the fluorophores [2,3]. Alternatively, the optical power of the excitation laser could be increased; however, this may worsen the photobleaching problem of the dyes [4].

The fluorescence imaging technique that uses a lower dose of fluorescent dye and/or lower excitation laser power is highly desirable for the long-term study of single cells. Hence, the use or development of a fluorescence signal acquisition method that has a high signal-to-background ratio (SBR) is essential. To this end, several digital lock-in techniques, which can extract a periodical signal from noisy measurements, have been proposed for charge-coupled device (CCD)-based fluorescence microscope systems. Fisher et al. extracted the fluorescence signal in the Fourier domain using the fast Fourier transform (FFT) with a series of phase-locked fluorescence signals [5]. Marriott et al. applied a typical cross-correlation analysis to the modulated fluorescence signals, from which the threshold correlation coefficients were extracted and subsequently used to map the fluorescence image [6]. Despite their high imaging quality, these lock-in methods commonly require acquisition of the time-varying fluorescence intensity signal over several modulation cycles and the pixel-by-pixel processing of dataset; thus, an image acquisition time of a few minutes is required to obtain a background-free fluorescence image. This time-consuming approach may hamper the cell viability owing to the long-term exposure to a strong excitation light [7].

In this paper, we propose a high-speed and high-contrast fluorescence imaging technique that uses the integrating-bucket method, which is useful for the long-term study of biological cells. By inducing a sinusoidal intensity modulation to the excitation light source and by simply capturing the CCD image for each quarter of a modulation period, only the fluorescence emission signal modulated by the source could be extracted. This homodyne

* Corresponding author.
E-mail address: leebh@gist.ac.kr (B.H. Lee).

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optical lock-in approach to epi-fluorescence microscopy enables the extraction of a low-noise fluorescence map within a sub-second time duration.

2. The four-bucket method

The four-bucket method is a type of digital lock-in detection technique, which extracts only the periodically modulated signal from very noisy measurements. It rejects the signals having frequencies other than the modulated one and selectively determines the amplitude and phase of the signal modulated at a fixed frequency. Thus, the method offers the possibility to enhance the SBR of a fluorescence imaging system by reducing the background level originating from ambient light and/or the dark current of the camera. The fluorescence emission in a sample can be modulated by simply modulating the intensity of the excitation light and synchronously detected by a CCD camera. In general, since the fluorescence emission occurs within nanoseconds after the absorption of excitation light, only the fluorescence signals excited with a known frequency can be identified.

Fig. 1 shows the schematic diagram of the proposing four-bucket technique. Four CCD frames are captured during one modulation period of the excitation light. The modulated fluorescence intensity, detected on a pixel (x,y) of the CCD camera, can be expressed as

$$I(x, y, t) = I_{dc}(x, y) + \Delta I(x, y)\sin(2\pi f t + \phi_0), \tag{1}$$

where I_{dc} is the intensity of the unmodulated signal, ΔI is the amplitude of the modulated fluorescence signal, f is the modulation frequency, and ϕ_0 is the overall delay between the modulated signal and the camera trigger. Then, the four frames captured by the camera at a frequency of $4f$ are expressed as

$$E_1 = \frac{1}{4f}I_{dc} + \frac{\Delta I}{2\pi f}\cos\phi_0 + \frac{\Delta I}{2\pi f}\sin\phi_0, \tag{2}$$

$$E_2 = \frac{1}{4f}I_{dc} + \frac{\Delta I}{2\pi f}\cos\phi_0 - \frac{\Delta I}{2\pi f}\sin\phi_0, \tag{3}$$

$$E_3 = \frac{1}{4f}I_{dc} - \frac{\Delta I}{2\pi f}\cos\phi_0 - \frac{\Delta I}{2\pi f}\sin\phi_0, \tag{4}$$

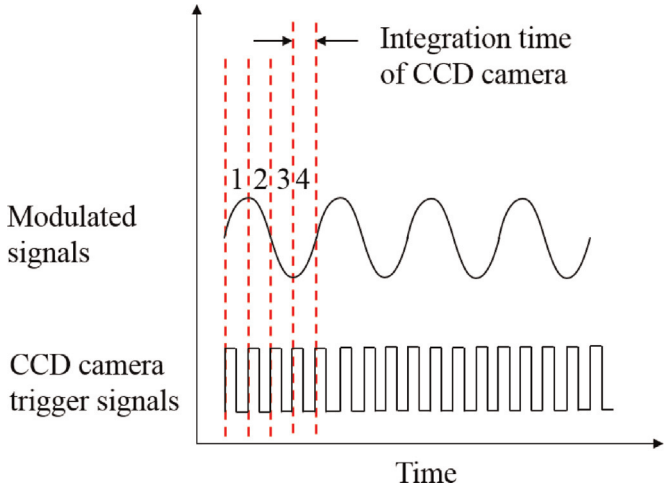

Fig. 1. Diagram of the image acquisition process. The numbers denote the time sequence of the frames acquired by the CCD camera. One image is calculated from four frames.

$$E_4 = \frac{1}{4f}I_{dc} - \frac{\Delta I}{2\pi f}\cos\phi_0 + \frac{\Delta I}{2\pi f}\sin\phi_0 \tag{5}$$

From these four frames, the amplitude of the modulated fluorescence signal can be simply extracted as

$$\Delta I(x, y) = \frac{\pi f}{\sqrt{2}}\sqrt{(E_1 - E_3)^2 + (E_2 - E_4)^2} \tag{6}$$

It is note that when the overall delay ϕ_0 is negligible, the 4 equations (Eqs. (2)–(5)) are degenerated into two, which means taking only on and off states are enough. However, to cope with general cases we assume delay ϕ_0, which is not negligible. The camera trigger can have a delay from the excitation, for example.

3. Experiments

The experimental set-up was similar to that of a typical epi-fluorescence microscope, as shown in Fig. 2. A 3 mW 405 nm laser diode (OZ-1000, OZ Optics Ltd.) was used as the excitation light source, and its intensity was voltage-controlled by a function generator (AFG3022B, Tektronix). The light from the laser was focused with a lens (L1) at the back focal plane of a water-immersion microscope objective (UNPFLN, 10× magnification, numerical aperture (NA) 0.3, Olympus) via a dichroic mirror (DMLP425R, reflection/transmission wavelength: 405/450 nm, Thorlabs, Inc.). The laser illuminated the sample through the objective with an average incident power of 365 μW. The fluorescence signal emitted from the sample was directed to the dichroic mirror and was captured by a scientific CCD camera (COOL-1300, 512 × 640 pixels, 12 bits, VDS Vosskuhler GmbH) through the tube lens (L2). A 450 nm bandpass filter (MF460, Thorlabs, Inc.) was

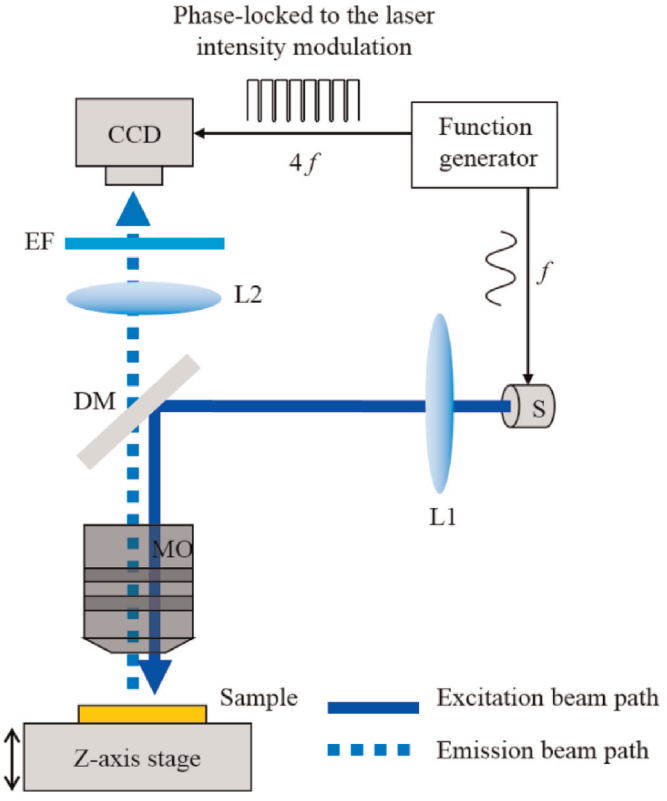

Fig. 2. A schematic of the epi-fluorescence microscope with a homodyne lock-in configuration with EF: emission filter; L1-2: lenses; DM: dichroic mirror; CCD: charge-coupled device; MO: microscope objective (water-immersion, 10× magnification, NA 0.3); S: excitation laser source (405 nm laser diode).